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REVIEW ARTICLE

Assessing fibrous fractions in feedstuffs: A review of analytical techniques

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ABSTRACT

This review examines key analytical methods for determining the nutritional value of forage and feedstuffs, focusing on the fibrous fraction's variability and its impact on animal nutrition. Traditional and modern techniques, including the widely used detergent system and enzymatic-gravimetric methods, are evaluated for their accuracy, reproducibility, and practicality. Despite improvements, challenges remain due to methodological variations and the high costs of advanced techniques. The review highlights the need for further research to enhance analytical methods, ensuring precise estimation of cell wall constituents for optimal diet formulation in animal production.

Keywords: alternative methods; nutritive value; Van Soest; source of variation

1. Introduction

The estimation of the nutritive value of foods for diet formulation involves a series of studies that evaluate mainly the fibrous fraction. Since this presents great variability and is directly related to energy availability and consumption[1,2].

In Brazil, where food resources are based on grasses, the knowledge of the nutritional value of food is of great importance for the understanding of physiological processes, since they are responsible for the transformation of food into animal products.

Fiber is a nutritional term that can only be determined during the digestive process and its definition is linked to its origin and the analytical methodology used in its laboratory determination[3,4]. Resistant to enzymatic digestion, fiber ends up serving as a substrate for fermentative activity[5,6] by presenting variable digestibility, it occupies space in the gastrointestinal tract and can limit animal consumption[1]. Thus, errors made in laboratory analysis for estimating the nutritive value of feedstuffs can compromise the balance of diets[5].

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The traditional analytical method called detergent system, developed by Van Soest (1963b), for the quantification of insoluble fibrous compounds, underwent a series of modifications in certain stages of its process in an attempt to improve its use to cover a greater variability of foods. These modifications suggested the withdrawal of decalin used as an antifoaming agent; the withdrawal of sodium sulfite used to remove protein contaminations; the exclusion of sodium sulfite and the inclusion of α-amylase for amide removal[6]; the use of α-amylase, sodium sulfite and triethylene glycol without decalin[5].

Due to the various alterations made to the original method, in 1980 David Mertens, in order to reduce errors among laboratories, created only one single analytical method for all types of foods[7,8].

However, even if all researchers rigorously adopt the established reference method, there are still some practical shortcomings that may end up limiting its efficiency in terms of comparing results[2,7]. There are many factors that cause variations in results between laboratories, such as, for example, adaptation of methods to increase throughput or even adaptation of equipment limitations or laboratory conditions. In addition, it is important to emphasize that the analytical results obtained generally indicate only the content of a feed and not the nutritional value that the animal will obtain by eating, digesting and metabolizing it[1,3].

As a way to minimize these variations, Mertens (2003)[3] cites that the different methods need to present inference and reproducibility. Inference refers to the way in which the method will provide correct information and promote an adequate description of the food and reproducibility refers to the ability of the method to be reproduced in duplicate in different laboratories, presenting similar results for the same analysis using the same food, thus ensuring accuracy and precision of the results.

In this context, even though the method for obtaining the fibrous components has been standardized by Mertens (2002)[9] and indicated by the AOAC as a reference method. There are more current analytical methods, which differ from each other. The analysis of the fibrous components without the exclusion of possible errors associated with these differences can compromise their use, making it difficult to compare the results. According to Mertens (2003)[3], the acceptance of an analytical method is based directly on its capacity to combine the concept of fiber and its reproducibility among laboratories.

The most current methods present a limiting factor for many laboratories, and it refers to their accessibility and cost of equipment, making their use as a method of routine analysis difficult. It is also important to point out that many of these methods are not officially recognized, making the detergent system proposed by Van Soest (1963b)[10], even with its modifications, the most widely used, even though it does not determine exactly the composition of the cell wall components.

In this context, more research should be carried out in order to eliminate possible errors in the analysis, to improve laboratory methods, and consequently to obtain results that present precision (Jung, 1997)[1] and accuracy according to the citation of Mertens (2003)[3].

2. Definition and constituents of the fiber

Fiber is a nutritional term, which can only be determined during the digestive process and its definition is directly linked to its source and analytical methodology used in its laboratory determination[3].

Chemical, enzymatic or gravimetric laboratory methods were developed to quantify a fraction resistant to digestion and not a plant substance recognizable in plants, in addition to the division of plant fractions based on their structures and chemical bonds. Thus, its accuracy and relevance is totally dependent on the analytical methodology used[3].

Despite the divergences related to its definition, fiber originates from the cell wall of vegetative tissues, constituted by a set of highly complex and heterogeneous fractions[11], which are a mixture of polysaccharides frequently associated with other components, making them resistant to enzymatic digestion, but serving as a substrate for fermentative activity[1,5].

Fiber is classified according to its physiological state, water solubility and fermentation degree: insoluble fiber and soluble fiber, the insoluble fraction corresponds to the insoluble fractions in neutral detergent solution as cellulose and hemicellulose, which associated to lignin constitute the fiber in neutral detergent; the soluble fraction corresponds to the soluble fractions in neutral detergent, although more heterogeneous than the insoluble fraction, constituted by organic acids, simple sugars, amido and frutosans, plus pectic substances[12,13].

Cellulose is the most abundant polysaccharide in the cell wall of plants and its proportion varies from 20% to 40% on a dry basis[11,14]. Constituted by long linear chains of D-glycose, linked by β-1,4 bonds with a high degree of polymerization and molecular weight. With a fibrous structure of crystalline character, without branches and with rigid and inflexible conformation, it presents high resistance to breakage by different chemical substances[11,15]. These linear chains are linked together by hydrogen bonds and Van der Waals forces, being called cellulose microfibrils. They are compacted and are responsible for the mechanical resistance of the tissues they compose, which is important in the evaluation of forage plants. Because this association can influence the sensitivity of the cellulose molecule to microbial enzymatic hydrolysis[15,16].

Hemicellulose is associated with cellulose in the cell wall, in the intramolecular spaces of cellulose microfibrils, acting as a mass because of the way they connect with the other structural components. Hemicelluloses can be both linear and branched polymers, made up of amorphous polysaccharides with a lower degree of polymerization than cellulose[15]. Plants in a more advanced stage of maturity are more closely associated to lignin by covalent bonds than to other polysaccharides, becoming unavailable for solubilization. These present great variation among the types of hemicelluloses and vegetative species, constituting about 10% to 25% of the dry base of forage plants[15]. Hemicelluloses are divided into four subgroups, also called glycans, which are linked to the microfibrils that make up the cellulose through hydrogen bridges, showing structural differences[16].

Pectins are present in large quantities in cell walls and intracellular tissues of young tissues in the form of protopectins, contributing to rigidity, transport and water retention. Constituted by galacturonic acids that present covalent bonds of the α-1,4 type, intercalated with rhamnose, arabinose and galactose units with chain branches of pentose and hexose units, differing from each other in terms of esterification degree and number of methoxylations. These substances are divided into: pectic acids without methoxylations; pectinic acids with methoxylations that may or may not be water-soluble; and pectins, a subgroup of water-soluble acids[17,18].

Lignins are complex polymers of little known structure and chemically linked to cellulose and hemicellulos. Constituted by condensed polymers, of different phenylpropanoid alkoxys whose precursors are p-coumaric, ferulic and sinapic acids, which will condense through an oxidative process forming cross-linked

macromolecules, the lignins[1,11,17]. Lignin is extremely resistant to chemical degradation, provides rigidity to the cell wall, structural support and physical resistance to plant tissues. It has a condensed structure, through carbon-carbon covalent bonds and ether or ester bonds. Due to these bonds, it becomes extremely resistant to the hydrolytic action of acids and bases; moreover, its composition, structure and quantity are variable in plant tissu[1,11,17]. According to[10], this resistance delays the development of laboratory techniques for its quantification.

The fiber constitution is quite variable and depends on several factors. Mainly factors associated with the plant species, between varieties, within the same species and between organs or tissues, depending on the stage of development and/or maturity of the plant tissue[1,8,18].

Due to this variation, many analytical proposals are found in the literature for the determination of fibrous fractions, and they are divided into two groups: gravimetric and enzymatic-gravimetric (**Table 1**).

Table 1. Gravimetric and enzymatic-gravimetric methods. Methods analysis authors.

Methods	Análises	Authors
Gravimetric	Crude fiber	Weende (1859)
	Fiber in neutral detergent and Fiber in acid detergent	Van Soest (1963)
	Acid detergent fiber	Goering & Van Soest (1970)
	Neutral detergent fiber	Van Soest & Wine (1967)
Enzymatic-gravimetric	Enzymatic neutral detergent fiber (eNDF)	Van Soest & Robertson (1985)
	Amylase-treated and amide-free fibrous organic material (“aFDNmo”)	Merntes (2002)

3. Determination of fibrous fraction, methodological alterations and limitations of use

According to[19], Einhoff in 1809 was the first researcher to try to separate the fibrous fraction of food, through the maceration of fibrous material in water, filtration and weighing of the residue. In 1859, the method known as proximal analysis system or Weende method for crude fiber determination, was standardized in the Experimental Station of Weende[6]. This method consists of two consecutive extractions and the food is treated with two solutions, one acidic and the other alkaline; in the first extraction with the use of a diluted acid solution there is removal of amide, simple sugars, part of the pectin and hemicellulose; and in the second extraction with the use of a diluted alkaline solution there is removal of proteins, pectins, part of the lignin and remanescent hemicellulose. The final residue is washed with quente water and alcohol, dried in an oven, weighed, finally incinerated and determined to cinza. The crude fiber is determined by subtraction of the cinza to the final residue[1,18].

Subsequently, the Wennde method was challenged, once after the analysis of the residues, it was verified that the crude fiber is constituted of cellulose with small amounts of lignin and hemicellulose. As a result, the method became limited, due to the solubilization of lignin, an indigestible component[19] and also of hemicellulose[10], a fibrous carbohydrate. According to[10] the amount of lignin solubilized in this method is removed in the second extraction by the alkaline solution together with hemicellulose, causing these fractions to become part of the non-nitrogenous extractive fraction. And the digestibility of the non-nitrogenous extractive becomes inferior to that of crude fiber mainly due to the presence of lignin[1,18,19].

Due to the above mentioned problem[20], developed the technique known as detergent system, with the intention of isolating the fibrous fraction and developing a more effective method to replace the crude fiber method. This was initially developed to isolate insoluble dietary fiber and plant cell wall constituents such as cellulose, hemicellulose and lignin[6,8,21].

Also in the 1960s, in the USA, the American organization AOAC (Association of Official Analytical Chemists) made official the Weende method for the determination of crude fiber, even with the above-mentioned limitations. In 1963 the AOAC recognized the method known as the Acid Detergent Method for the determination of fiber and lignin developed by Van Soest, since it was not difficult to standardize it among chemical analysis laboratories[1,7,8]. This method quickly replaced the Weende method, since it was characterized as a simpler method and resulted in values similar to those found by the Weende method.

However, according to Mertens (2003)[3] the acid detergent methodology is used to minimize lignin losses, thus it does not fit into the nutritional definition of dietary fiber. Because the hemicelluloses in acid detergent are removed and pectin, which is a rapidly fermentable carbohydrate, is not removed, it becomes an inadequate method for estimating the fibrous fraction. Possibly the precipitation of pectin in this method could be the reason why some foods with high amount of pectin show results of fiber in acid detergent higher than fiber in neutral detergent.

In 1967a, Van Soest & Wine[22] developed the Neutral Detergent method, where the results found for crude fiber were superior to those obtained until 1967, because it allowed greater isolation of the cell wall fractions, the acid detergent fiber methodology became less interesting for fiber determination.

According to Van Soeste (1967b)[21] the acid detergent fiber methodology was developed as a preparatory analytical step for lignin determination and has never been used to measure fiber in food. In addition to assisting in the estimation of lignin, the acid detergent methodology allows the determination of celluloses, insoluble nitrogen in acid detergent and insoluble zinc in acid and silica[7]. Fiber in acid detergent is determined by refluxing a sample in solution containing sulfuric acid[9,21].

The determination of neutral detergent fiber content is based on the solubilization of the cell wall of the food and through filtration the cell wall is separated from the cellular content. However, part of the pectin ends up being solubilized and quantified as cellular content, thus becoming a frequent routine in food chemical analysis laboratories[2].

Neutral detergent fiber is characterized as a fraction that occupies space in the gastrointestinal tract and has inconsistent digestibility[9] and can affect consumption, presenting the need to reduce the size of its particles through mastication to facilitate digestive processes[3]. The soluble content in neutral detergent is highly digestible and totally fermentable in the rumen[19,21], occupying little space in the treatment[1,3]. It is characterized as a standard fraction because it allows the separation of its fractions during ingestion and digestibility.

The system proposed by Van Soest used the concept developed by Lucas of ideal nutritional entities, in which the purpose was to identify the uniformity of the chemical fractions that constituted the food, true digestibility and constant endogenous loss. The same authors cited that if the food was analyzed by this concept, its nutritional value could be derived from the product's soma of each nutritional entity[16].

However, Van Soeste (1967b)[21] demonstrated that the cell wall of food does not behave in a uniform manner as the proposed concept, which makes it impossible to use a single fraction to predict the digestibility of dry matter. Even though the neutral detergent fiber is not uniformly presented according to the recommended concept, the soluble content in neutral detergent can be considered an ideal and uniform nutritional entity because it is almost completely available (98%)[16]. In addition, in this detergent system the food is divided into soluble fraction, which is quickly and completely available, and insoluble fraction, which is slow and completely unavailable.

In the original method of determination of fiber in neutral detergent, solutions with several reagents are used, each one having a specific function. Thus, a neutral detergent solution consisting of sodium tetraborate, ethylene diamine tetraacetic acid (EDTA), sodium hydrogen phosphate, sodium lauryl sulfate and triethylene glycol is used. Buffer solutions based on borate and phosphate are used to keep the pH close to 7.0 to avoid the solubilization of hemicellulose and lignin; sodium lauric sulfate and sodium sulfite to remove proteins; EDTA acid, being a chelating agent, helps in the solubilization of proteins and pectins and triethylene glycol for the solubilization of amides. Meanwhile, the method recovers cellulose, hemicellulose and lignin, with some contamination by proteins, pectins and amide. Associated with the washing of the fibrous residue with quente water for removal of the non-fibrous matter, followed by the use of acetone for complete removal of lipids and pigments in most of the samples[3,9,22].

Gradually, the original method developed by Van Soest & Wine underwent several modifications in one or another analytical step in order to open up a series of foods. Mainly those rich in amide and pectins, tannins and Maillard reaction products[5]. According to Van Soest et al. (1991)[5] and Mertens (2002)[9], the high values of neutral detergent fiber obtained in concentrated foods and forages could be associated with practical difficulties in the filtration stage, suggesting that the neutral detergent was not efficient in solubilizing mainly amide[12]. Thus, in 1982 Mongeau & Brassard recommended an alteration of the original method, through the inclusion of α-amylase, in order to eliminate amide. By making it gelatinized and hydrolyzed with amyloglucosidase, amylases have been used until now in the determination of neutral detergent fiber[5,23,24].

In the traditional method of Van Soest and Wine, monoethylene glycol ether was used for amide solubilization, but was replaced by triethylene glycol[5]. Decalin, an antifoaming agent, was withdrawn because it caused an increase in neutral detergent fiber values by removing lignin. Sodium sulfite was used for removal of protein contaminations, but it was removed from the methodology because it did not remove them completely and did not degrade lignin. Van Soest & Robertson (1985)[6] altered the original method by excluding sodium sulfite and including α-amylase for amide removal; the method of Van Soest et al. (1991)[5] kept α-amylase and recommended the alternative use of sodium sulfite, without decalin and with the use of triethylene glycol. The method of Mertens (2002)[9] recommends the use of sodium sulfite and α-amylase, in addition to the use of a reflux system with condensers for the extraction of cellular content, filtration and retention of insoluble residue in filtering chains.

David Mertens, in 1980 began to standardize the analysis of neutral detergent fiber among U.S. laboratories, concluding that the only way to reduce the errors among them would be through the analysis of all types of food from a single analytical method[7,8]. Thus, it was recommended that all foods should be analyzed using α-amylase, sodium sulfite and corrected for zinc. In addition, it was also determined that residual material such as amylase-treated and amide-free fibrous organic matter ("aFDNmo") should be defined as a nutritional entity [3,7-9].

Correction for blackheads allows elimination of errors arising from inadequate washing of the residues, and allows estimation of non-fibrous carbohydrates, by difference[9]. This allows the exclusion of cell wall constituents such as polysaccharides and pectin, which undergo rapid fermentation and digestion and are similar to the cellular content. Mertens (2002)[9], also recommends that the aFDNmo, besides being conducted with standardized solutions of α-amylase, should be white, to correct possible errors related mainly to sample weighing[13].

The Mertens method (2002)[9] was adopted as an official method by the Association of Official Analytical Chemists (AOAC, 2002), even with technical flaws, such as difficulties in filtration and removal of amide, protein contamination, leading to the inclusion of an enzymatic digestion with the use of α-amylase. This was

developed for all types of food, from forage plants, grains, oilseeds and animal agribusiness by-products. However, it presents methodological modifications when compared to the original and includes the addition of sodium sulfite to remove protein contaminations and α-amylase to remove amide contaminations during the extraction stage with neutral detergent. However, the use of α-amylase requires industrial standardization at each new analysis.

The methods developed and recommended by Van Soest et al. (1991)[5] and Mertens (2002)[9] are different. Van Soest et al. (1991)[5] cite that the use of sodium sulfite produces undesirable reactions, which would damage cell wall proteins and could solubilize part of the lignin. Já Mertens (2002)[9], cites that sodium sulfite does not solubilize lignin and its use is important for the removal of protein contamination.

Gomes et al. (2012)[4], evaluated the effects of the use of sodium sulfite in a neutral detergent solution on the estimation of fibrous composts in tropical grasses and legumes. They observed that the NDF value decreased when sodium sulfite was used, with a greater reduction in legumes. In addition, sodium sulfite reduced the FDF value in both forages. Lignin content was reduced by sodium sulfite in legumes, but no effect was observed in grasses. The decrease in fiber content in legumes can be explained by the solubilization of lignin. However, the decrease of fibers in grasses cannot be explained only by the decrease of contaminant protein and lignin solubilization, probably losses of other fibrous compounds occurred.

In summary, the analytical methods are developed based on a standard method. In the case of the crude fiber method, there is no specific standard method, only a reference method recommended by the AOAC, and it was developed and improved gradually based on an empirical method. Portanto, the method for determining fibrous fractions still remains empirical, and all the analytical steps detailed by Mertens (2002)[9] as well as any modification of the method may affect what is measured as fiber[4,13]. Thus, the results from the detergent system depend strictly on the recommendations described in the protocols.

Finally, it can be said that, although all researchers rigorously adopt the protocols, there are some practical limitations, which may end up limiting their efficiency in terms of comparing the results obtained[2,7].

In this context, the journal Animal Feed Science and Technology recommends the use of the article by Mertens (2002)[9] as the main reference for neutral detergent fiber analysis. For the analysis of acid detergent fiber, the use of the AOAC manual with the identification number of the specified procedure is recommended; and for the analysis of lignin and cellulose, the sequential quantification from the oxidation of acid detergent fiber in potassium permanganate solution and through the burning of the residue in a muffle, respectively, is recommended[1,7,8].

Of the three methods used to quantify fiber (neutral detergent fiber, acid detergent fiber and dietary fiber), only neutral detergent fiber is able to estimate the three major indigestible or incompletely digestible components of plants: hemicellulose, cellulose and lignin.

Even following the recommendations of the Journal Animal Feed Science and Technology, the detergent system presents practical limitations, related to the excessive work in the performance of the reflux and filtration stages of each evaluated sample, which ends up limiting its efficiency in the use of human, financial and even infrastructure resources within the laboratory[2,7]. Thus, the choice of the analytical method to be used will depend on some criteria, such as the objective of the researcher or the laboratory, since each method has positive and negative points (**Table 2**).

The conventional method, standardized by Mertens (2002)[9], suggests the use of a refluxing apparatus with condensers and extraction carried out in tanks, and the final residue retained in individual filtering boxes. In addition, there are methodologies that are conducted in totally pressurized environments such as the

Ankom® system, which is based on the digestion of conditioned samples in filter bags subjected to extraction with neutral detergent. Unlike the traditional method, this system is less laborious and allows the analysis of a large number of samples per day, where steps such as manual washing and filtering are eliminated. However, one of the drawbacks of using this system refers to the high cost of the bags used, which are obtained from the company that manufactures the equipment (F57 Ankom®)[2,24].

Thus, in order to reduce the cost of analysis using Ankom® bags, many studies have already been conducted with the use of bags made of similar fabrics such as nylon and tecido-nylon-tetrafluoroethylene (TNT). In order to evaluate the efficiency of using nylon, F57 (Ankom®) and TNT bags in the analysis of indigestible neutral detergent fiber (INDF) of samples with particle size of 1 mm, Casali et al. (2009)[4] observed that the INDF values found with F57 and TNT were higher than those obtained with nylon bags, due to particle loss. Valente et al. (2011b)[24], with the same objective, but different foods, cited that the use of F57 and TNT bags resulted in accurate estimates of NDF content, different from nylon bags also due to the loss of fibrous particles, underestimating the result.

Based on the results presented, it is possible to conclude that TNT bags become an alternative to replace F57 in reducing the cost of analysis. Lanes et al. (2016)[28] cites that possibly this particle loss is due to dilation of the nylon bag mesh during the reflux stage.

Berchielli et al. (2001)[2], following the standardization of the conventional method of Van Soest, compared the values of neutral detergent fiber (NDF) and acid detergent fiber (ADF) of different foods from the Ankom® equipment with the use of four types of saquinhos, and concluded that the different types of saquinhos did not influence the NDF values, with the exception of bovine feces, obtaining lower NDF results. No difference was observed between NDF and FDF values in the results found by the Ankom® equipment and the conventional method, except for citrus pulp, where the FDF by Ankom® was lower than that obtained by the conventional method.

Ferreira & Mertens (2007)[8] also followed the recommendations described by Ankom® and compared the results with the method of Merterns (2002). By evaluating the determination of NDF without the presence of sulfite, NDF with the use of α-amylase and aFDN (with the presence of sulfite and α-amylase) using two extraction methods: refluxing with filtering chains (Mertens, 2002) and Ankom®, in 33 samples of millet silage. They concluded that the absence of α-amylase in the Ankom® method overestimates the NDF values, possibly due to the gelatinization of the amide, which hinders the filtration of the residues. No differences were observed for aFDN concentrations in the two extraction methods, and the lower concentration of fibrous residue for NDF could be attributed to a lower protein contamination of the fibrous residue.

The objective was to compare the values of the acid detergent fiber factor (FDA) of 12 foods using different analytical procedures: two of them using the Ankom® technique, one with direct treatment of the sample in acid detergent (D-ADF) and another sequential treatment with neutral and acid detergent solution (D-ANDF); the third procedure being the conventional methods of Goering and Van Soest (1970) (VS). Danelón et al. (2013)[5], observed that the results found differed among themselves for all procedures (24.58%, 27.83% and 28.01%). The FDA theory found in the procedure with direct treatment of the sample was higher than the sequential procedure, with the exception of the millet. Possibly due to the removal of food fractions by the neutral detergent, such as pectic substances.

Table 2. Summary of the use and limitations of the main analytical methods used in the determination of fibrous fractions of forage plants. Adapted: SEGURA et al., 2007[8].

Gross Fiber	Part of the cell wall that survives digestion under acid and alkaline solubilization. Cellulose and lignin are recovered in large proportions.	Solubilization of lignin and hemicellulose. Overestimate or crude fiber value.	It consists of two consecutive extractions: in the first one, the food is subjected to an acid solution and the second to an alkaline solution. The residue is washed with hot water and alcohol, dry, heavy, by fim incinerated and determined to cinza. The crude fiber determined by subtraction of the mass of the cinza at mass of the final residue.
Neutral detergent fiber (NDF)	Fraction of food that is not completely digested, with almost complete recovery of the cell wall.	Partial recovery of pectins. Protein and amide soup.	Van Soest (1967): similar to Weende's method, only that is used chemical extractions with a detergent solution neutral over-refluxing. Mertens (2002): similar to the Van Soet method, with the inclusion of an enzymatic digestion through α-amylase.
Acid detergent fiber (ADF)	Cellular wall porpao.	Part of the lignin is solubilized. Pectin precipitation.	Van Soest (1967): similar to the neutral detergent method, only using chemical extractions with detergent solution acid over-reflux. Method of analysis approved by AOAC (2002).
FDN-FDA	Hemicellulose	Limitapóes dos métodos FDN e FDA.	FDN - FDA
Lignin	Lignin	Lignin solubilization in FDA methodology.	Sequential quantification from detergent fiber oxidation acid in potassium permanganate solution, and by means of the muffle residue burner.
FDA - Lignin	Cellulose	Limitapóes dos métodos FDA e Lignina.	FDA methodology. Sequential quantification from detergent fiber oxidation acid in potassium permanganate solution, and by means of the muffle residue burner.

Da Silva et al. (2018)[13], by comparing alternative methods for NDF analysis with the official method recommended by the AOAC in reflux system and filter cartridges, using 20 foods handled by three analysts. The alternative methods were: refluxing in beakers with beakers, Ankom® system, Tecnal® system and Micro-FDN (Autoclave). Regarding the variability within the laboratory for aNDFom, they observed that the effect of the method-food-analyst component was not significant for the analyst variability component. With significant interaction between method-food for aNDFom. Therefore, the effect of in-feed methods and vice-versa revealed significant contrasts between alternative and reference methods for aNDFom. The refluxing and filtering methods showed no differences, independent of the analyzed food. Significant contrasts demonstrated the lack of quality of the alternative methods used to measure insoluble fiber compared to the reference methods. Barbosa et al. (2015)[25], cite that the autoclave method can be substituted by the conventional method, because it generates accurate results.

In an experiment with the objective of comparing the results obtained by the FDN and FDA non-sequential and sequential analysis through the conventional method proposed by Van Soest et al. (1991)[5] and the alternative method using the autoclave with different bags (Ankom®, TNT and filtering chains). Lourenço et al. (2017)[21], observed that the accuracy of the analysis of NDF and FDA values did not show differences between non-sequential and sequential analysis, in all the foods and methods used, with the exception of the determination of FDA in millet silage, possibly due to the high amide content that was gelatinized inside the filter during the boiling process. Thus, the analytical precision of the alternative methods as well as their use, when compared to the conventional method, depends on the analyzed food.

When comparing the use of alternative equipment such as the fiber digester and the autoclave with nylon (50 μm) and TNT (100 g/m^2) bags in the determination of NDF and FDF to the conventional method, Farias et al. (2015)[26] concluded that the NDF value obtained with TNT fabric in the two equipments did not differ

from those found by the conventional method. However, the use of nylon fabric in both equipments was similar, indicating a possible alternative to the conventional method. There was no difference between the alternative methods of NDF analysis. The FDF theorems differed among the equipment and fabrics evaluated. However, the values obtained with the nylon fabric in the digester equipment were similar to the conventional method, which could be a practical alternative for FDF analysis. The NDF and FDF values obtained in the fiber digester equipment with nylon fabric were similar to the conventional method.

In this context, the modifications made to the conventional method of Van Soeste and Van Soeste & Wine are different from each other and have advantages and disadvantages. In Brazil, the vast majority of published articles use the methodology described by Silva & Queiroz (2002)[27], which is based on the methodology recommended by the AOAC. Lanes et al. (2016)[28], evaluated the NDF and FDA values in bovine food and feed samples. Through the conventional method, following the recommendations of Silva & Queiroz (2002)[27], and the automated method with TNT sachet (Tecnal® 149). They concluded that the automated method did not differ from the conventional method in terms of NDF concentration in tropical forage, fezes and millet silage samples. It was not efficient for samples with high amide content, even when using α-amylase.

Geron evaluated the NDF and FDA values of four capins using three methods: the conventional method, the Ankom® method and the method adapted by EMBRAPA. The authors observed that the NDF and FDF values of the capins obtained by the different procedures did not differ from each other. They recommended the procedure adapted by EMBRAPA, since it did not differ in relation to the conventional and Ankom® methodologies, in addition to having lower reagent costs and consequently lower cost. It should be noted that the authors used the recommendations of Silva & Queiroz (2002)[27].

The research presented here is aimed at obtaining alternative methods of analysis that are more precise and accurate, more economical and that reduce the work time when compared to the conventional method, which has a series of laborious steps. Thus, the neutral detergent system will continue to be empirical and its accuracy, reproducibility and inference of its results depend on the rigorous conduction of all the analytical steps, in order to produce comparable and acceptable results regarding the fiber quantity according to its definition.

4. Analytical problems and implications of the methodologies used in determining the components of cell parade

According to Jaurena et al. (2012)[29], the occurrence of errors in the interpretation of food chemical analysis results is becoming increasingly common. Thus, it is important to emphasize once again that there is a need for standardization of the terminologies used in analytical methods (Udén et al., 2005)[30].

Mertens (2003)[31] cites that before discussing any existing analytical variation, it is important to highlight some critical points, such as: all analytical results are only percussors of the real nutritional value of a large quantity of food; variability may be natural and unavoidable; variation can be divided into precision and exaggeration; reproducibility and statistical inference are necessary to detect differences and provide adequate confidence intervals for the results. However, it is important to note that currently one of the major problems related to analytical variation is the selection among the several existing possibilities of the best method to be used to determine the chemical-bromatological composition of the food (Hall, 2007)[12].

The results from food evaluation are vulnerable to any variation, however, the intrinsic and extrinsic variation of the quality of the food used in the formulation of diets causes a greater variability of the analytical results. The sources of intrinsic variations refer to the food's own characteristics that differentiate it from others and are related to physical, chemical and nutritional properties. Extrinsic variations are those foreign to the

nature of the food and are associated, for example, with sampling, analytical procedures and the quality of the reagents used (Jaurena et al., 2012)[16].

In this regard, it is important to consider the first critical point cited by Mertens (2003)[31], that all the results obtained are only predictors of the real nutritional value of a large amount of food. Probably, the main problem consists in the fact of carrying out with precision a representative sample of the material to be analyzed. Associated with the sample, it is worth noting that the sample sent to the laboratory will be ground to obtain particles with a size of 1 mm and only a parcel of this (1 g) will be analyzed (Mertens, 2002)[9]. However, even if more than one laboratory analyzes a plot of the main sample, it is possible that the results found will be different. Since the plots are not the same, the second critical point above can be established, where variation is normal and unavoidable. Due to the small amount of sample analyzed, it is likely that the result found is only an adequate estimate of the average composition of the whole, provided that the initial sample and the analysis are performed correctly. Thus, the sample of the material to be analyzed is probably the most important source of error in analytical methods (Undersander et al., 1993)[32].

Valente et al. (2011a)[33], in order to evaluate the influence of particle size (1 and 2 mm) on NDF values using nylon bags, F57 (Ankom®) and TNT. They concluded that the wetting should be carried out using 1 mm porosity pencils for proper extraction of the cellular content by neutral detergent and efficient action of the thermostable α-amylase enzyme, since the use of 2 mm particles led to an overestimation of the NDF values. Regarding the material used for sample conditioning, the results showed that F57 and TNT fabrics provided accurate estimates of NDF values. In turn, the accuracy of the results obtained with nylon fabric was compromised due to particle loss. Based on the results, it was expected that the samples wetted at 2 mm would overestimate the NDF values, because the surface area of the sample was so much greater for penetration of the neutral detergent when compared to the 1 mm samples. The opposite is also valid, wet samples with particle size smaller than 1 mm can be washed and lost in the filtration stage. In addition, particles larger than 2 mm can clog the filter membrane, making the filtration stage more difficult.

Even with the occurrence of possible errors associated with the stages of preparation of the sample to be analyzed, Mertens (2003)[3] and Hall (2007)[12], cite that variation can be divided into precision and accuracy, associated with reproducibility and statistical inference to detect differences and provide adequate confidence intervals for the results. Accuracy refers to the absence of variation between the results of the same analysis and the same food between laboratories. The accuracy is related to the result, which must be authentic or true (closer to the real).

In order to find accurate results, it is important that laboratories perform their analyses in duplicates, to obtain an average result closer to the real value. According to Undersander et al. (1993)[32], there are standard deviation values that should be accepted when performing duplicate analyses, but if the values found are higher or lower than the real value, it will be necessary to perform new laboratory analyses.

However, there is an important factor that can affect the variation of the analysis and refers to the heterogeneity of forage plants and their fiber, as well as the heterogeneity of other foods used in the diet formulation of ruminant and non-ruminant animals. The constituents of forage plants such as leaves and grass, for example, have different compositions independent of their physiological state, and a true result of the chemical-bromatological composition for comparison of the results will depend on a sample and a representative parcel of the whole. For this reason, analyses performed in duplicates or even triplicates allow additional information to be obtained to detect possible differences by calculating the mean and standard deviation of the sample.

Mertens (2003)[3] cites that reproducibility is the ability of the method to be reproduced in duplicates in several laboratories, with similar results for the same analysis using the same food, ensuring accuracy and precision. The inference already refers to the way in which the method will provide correct information and promote an adequate description of the food. According to Jaurena et al. (2012)[29], the variation coming from two laboratories has two specific causes: intra-laboratory variability, which refers to the variation existing within the same laboratory; and interlaboratory variability, which refers to the variations observed between different laboratories.

Probably, this variability may be related to small routine errors, differences between equipment and reagents used, lack of equipment calibration or even differences observed in the plots that are removed from the main sample sent for analysis that is not completely homogeneous. However, methodologies can be adapted to increase laboratory efficiency and save time. However, part of this variability can be reduced through the rigorous use of protocols, training of analysts and their familiarization with the methodology used.

It is also worth noting that, currently, this variability is easily observed through static analysis, where the errors related to the analytical methods are easily detectable by comparing the results found to those coming from the reference method.

Mertens (2002; 2003)[3,9] cites that for a method to be considered ideal, the interlaboratorial variation must be equal to zero and the intra-laboratorial variation must be equal to the variation between analyses from duplicates or triplicates. These results are verified through voluntary collaborative studies conducted by AOAC and National Forage Testing (NFTA), with the aim of quantifying analytical variations and establishing acceptable reproducibility by generating reproducible and comparable analytical results for rotational analysis. It involves about 8 or more laboratories, which analyze about 5 samples in duplicate through the routine methodology used in them. The results are then subjected to three statistical analyses and verified by the organization responsible for the collaborative study.

Hristov et al. (2010)[34], in a collaborative study to evaluate the variability in aFDN analysis, found a high variation in the analytical procedures used among laboratories, possibly due to the lack of rigorousness in following all the steps of the available protocol. They evaluated the variability for PDN analysis among 14 participating laboratories, through the use of the traditional reflux system and the Ankom®. They concluded that the results of aFDN did not differ among the 14 participating laboratories, but differences were observed among the laboratories within the same technique and a high incidence of outliers for the Ankom® method. They emphasized the need for laboratories to follow exactly the protocol of the official method, and recommended that laboratories carry out quality controls of the equipment and procedures used.

In this context, it is important to note that all available analytical methods for the determination of cell wall constituents are empirical (Mertens, 2003)[3]. Fiber is defined by the method of analysis and its source, so any modification in the analytical method can determine a new fiber value that is not comparable with that coming from the conventional method (Hall, 2007)[12]. Thus, the results from laboratory analysis present some indeterminacy, due to errors associated with the critical points cited by Mertens (2003)[3]. These can be minimized by improving the aspects related to the methodologies and equipment used and personnel training, as well as the exaggeration in the use of the protocols so that the results present reproducibility and repeatability.

Associated with these improvements, it is important to carry out more research, with the aim of developing new analytical methods that provide reliable results and a more rigorous laboratory routine.

5. Final considerations

The use of analytical methods allows estimating the composition and availability of the different fractions of the cell wall, but the incorrect interpretation of the results and the variability of the cell wall constituents require knowledge of the different analytical methodologies.

However, traditional or alternative analytical methods are still empirical. Considering that a negative point regarding the large number of analytical methodologies, which present different results for the same analysis, is the analysis and comparison of the results, since they are based on the variability of their own data to infer how much better or better is their result when compared to an intra- or interlaboratorial reference value or standard. In this context, this reference value or standard is derived from the traditional method, which ends up becoming "bad" when there is a very large variation of results between analytical methods. These can be minimized by the improvement of methodological procedures, equipment used and personnel training. In addition to the exaggeration in the use of the protocols so that the results present reproducibility and repeatability.

Alternative analytical methodologies that exhibit acuracy, reproducibility and repeatability when compared to the traditional method of Van Soest, Van Soest & Wine or the Mertens reference method appear to be viable in determining the constituents of the fibrous fractions.

Thus, the improvement of analytical methods is extremely important for the estimation of the nutritional value of foods. The limitations of analytical methods seem to be related to the inability to adequately solubilize the soluble fractions and part of the insoluble fractions, which end up being partially solubilized.

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Conflict of interest

The authors declare no conflict of interest.

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